Supporting information

Evidence for distinct electron transfer processes in terminal oxidases from different origin by means of protein film voltammetry

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Materials and methods

Protein purification

Cytochrome aa_3 and ba_3 samples were prepared as previously described.[1,2]

Preparation of the gold colloid

The preparation of the gold nanoparticles were performed following the method proposed by Turkevich[3] and refined by Frens.[4] In summary, 1 mM solution of $HAuCl_4$ was allowed to boil before adding 13 mL of a 39 mM aqueous solution of sodium citrate. After appearance of the red dark color, the solution was maintained under boiling for 10 minutes before cooling to room temperature. The gold colloid was centrifuged at 10000 rpm for 30 min and 95% of the surnatant was removed.

Electrochemistry

A 2 mm polycrystalline gold electrode was polished with 1 µm and 0.5 µm diamond paste and subsequently activated in a 0.1 M H_2SO_4 solution by maintaining the potential at +2.21 V for 5 s, -0.14 V for 10 s and then cycling 100 times between 0.01 V and 1.71 V at 4 V/s. A last scan between 0.01 V and 1.71 V at 0.1 V/s was done to verify the cleanliness of the surface. Then, three drops of 3 µL gold colloid were deposited successively on the electrode surface and allowed to dry under air. The surface area of the newly modified electrode was estimated by integration of the reduction peak at 1.1 V assuming a value of 390 $\mu C.cm^{-2}$ for a gold oxide surface. The electrode was then immersed overnight in a 1:1 solution of mercaptohexanol and

hexanethiol. This functionalization was favored because the proteins of interest are known to have a largely hydrophobic surface and thus give more stable films on hydrophobic surfaces. To immobilize the protein on the modified surface, however, the concentration of the detergent (dodecyl maltoside) in the sample was kept below 0.1%. 20 μL of a protein stock solution was therefore washed once with 200 μL of 50 mM KPi buffer in an Amicon Ultra 50 kDa. The electrode was then rinsed with fresh solvent, 3 μL of 100 μM protein solution were deposited on the surface and left at 4°C overnight. Finally, the modified electrode was rinsed to remove the excess of protein not adsorbed on the surface. All the measurements were performed in a standard three electrode cell connected to a Princeton Applied Research VERSASTAT 4 potentiostat. An AgCl/Ag 3M NaCl reference electrode was used together with a platinum wire as counter electrode. The potentials mentioned in the article and table S1 are referenced to the standard hydrogen electrode. The anaerobic measurements (see figure S1) were performed by degassing the buffer solution for at least 30 min under Ar and by preparing the cell in a glove bag. The electrode was then transferred to air-equilibrated buffer solutions for the electrocatalytic studies. All the measurements shown in the main manuscript were carried out with a rotating speed of 1000 rpm and a scan rate of 0.02 V/s.

For the pH-dependent studies (figure 1), the proteins were equilibrated in the respective buffer solutions prior to deposition on the surface of the electrode. For comparison purposes, the voltammograms shown in figure 1A and 1B were normalized to the same surface area. In order to obtain the turnover frequency values (k_{cat}), measurements were carried out at different electrode rotation speeds and the reciprocal limiting currents $1/i_L$ were plotted versus the reciprocal square root of the electrode roration speed $1/\omega^{0.5}$ (Koutecky-Levich plots, figure S2). These plots give access to the reaction-rate-limited current (i_{cat}) at infinite rotation rate.[6] Under substrate

saturation conditions, k_{cat} can be determined by using the simple formula $\boldsymbol{k_{cat}} = \frac{\boldsymbol{i_{cat}}}{\boldsymbol{nFA\Gamma}}$ where n is the number of electrons involved in the catalytic reaction, F the Faraday constant, A the surface of electrode and Γ the active protein surface coverage. Γ can b estimated by integration of the anodic or cathodic signals in absence of oxygen (figure S1). Typical k_{cat} values obtained for the immobilized proteins are in the range of 1 to 10 s^{-1}. These values are lower than those obtained by spectrometric methods in solution (100 to 1000 s^{-1}), probably due to low interfacial electron transfer rates.

The temperature-dependent studies (figure 2) were carried out by dipping the modified electrodes in pH 7 buffer solutions equilibrated at the respective temperatures.

Fourier Transform Infrared Spectroscopy

To perform the infrared spectroscopy experiments, samples needed to be prepared in D_2O solution since H_2O shows a strong absorption in the Amide I region. The buffer exchange was done by washing 3 times 20 µL of the sample with 200 µL of 50 mM phosphate buffer prepared with D_2O (0.02% DDM, pD 7) in a Amicon Ultra 50 kDa membrane. Then, the samples were kept at 4°C for 12h to finalize the H/D exchange. After that, the protein was introduced in a transmission cell connected to a thermostat. Spectra were taken every 10°C between 5°C and 75°C. At each temperature step, spectra were recorded until the signal was completely stable. Spectra were obtained using a Bruker Vertex 70 spectrometer with an accumulation of 256 scans at a resolution of 4 cm^{-1}.

Characterization of the modified surface by Surface-Enhanced Infrared Spectroscopy (SEIRAS)

To characterize the modified surface, SEIRAS spectra were taken for each modification step using a Bruker Vertex 70 modified with a Harrick ATR cell. Each spectrum recorded corresponds to 256 averaged scans at a 4 cm^{-1} resolution. A silicon internal reflection element from an ATR cell unit was modified with a 20 nm thick gold layer of a gold substrate by sputtering. Then, 3 µL of the gold nanoparticles solution were deposited on the surface and allowed to dry under air. The SEIRAS spectrum shows two characteristic bands of the citrates which stabilize the gold colloid at approximately 1600 cm^{-1} and 1400 cm^{-1}. Then, 10 µL of a 1 µM solution of mercaptohexanol and hexanethiol in EtOH were deposited on the surface. After 30 min and removal of the solution, the surface was rinsed with ethanol to eliminate the non-specifically adsorbed species. The bands characteristic of the citrates now appear in negative while two bands specific of the C-H stretching modes of the thiols can be seen at approximately 2930 cm^{-1} and 2860 cm^{-1}. Finally, 3 µL of a 100 µM sample of protein were added on the surface. After adsorption of the enzymes, the excess of non adsorbed protein was removed by several washings with fresh buffer. The characteristic Amide I band (between 1700 and 1600 cm^{-1}) and Amide II band (between 1600 and 1500 cm^{-1}) can be observed. In all studied samples, the position of the Amide I band is typical for a protein with predominant α-helices contributions. There are thus no major changes in the secondary structure of the protein after immobilization. The Amide I/Amide II ratio[5] was determined as described by Todorovic et al.. Immobilization of cytochrome aa_3 yields to an Amide I/Amide II ratio of approximately 3 whereas it is near 2 for cytochrome ba_3. These results could suggest that cytochrome aa_3 adopts a more perpendicular orientation on the surface than cytochrome ba_3.

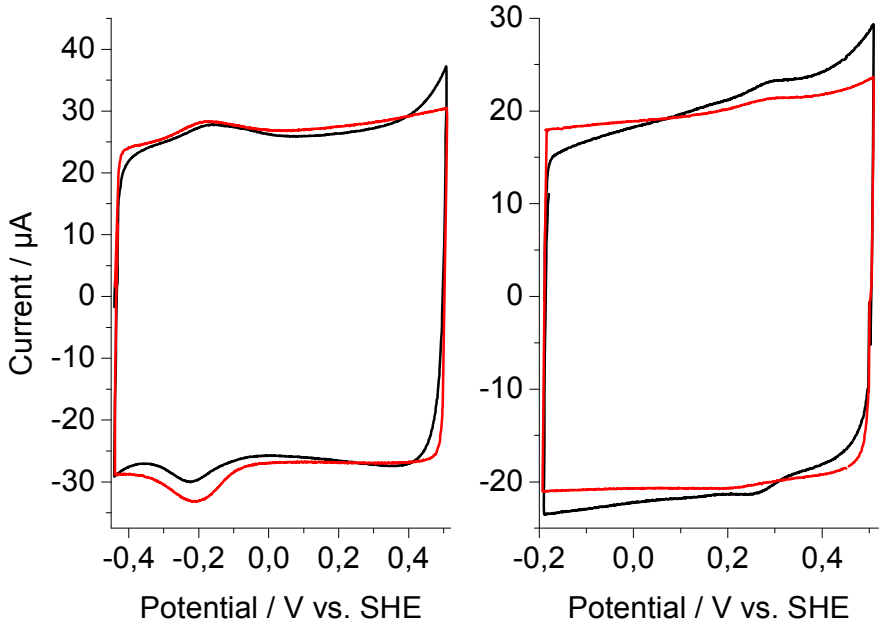

Figure S1. Voltammograms under non-catalytic conditions obtained for cytochrome aa_3 (A) and cytochrome ba_3 (B) (v=0.5 V/s)

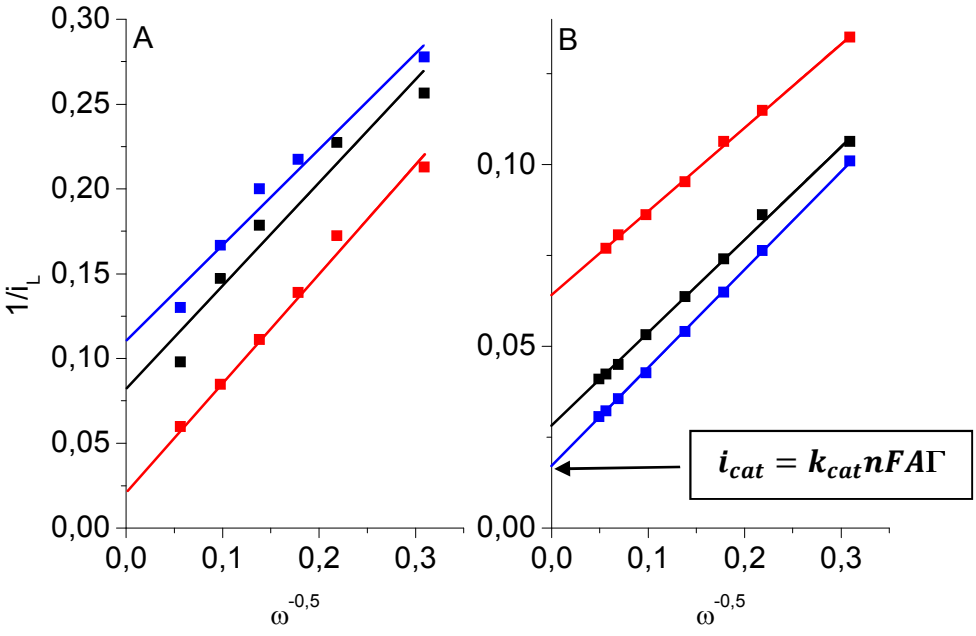

Figure S2. Koutecky-levich plots obtained for cytochrome aa_3 oxidase (A) and cytochrome ba_3 oxidase (B) at pH 6.5 (black), 7 (blue) and 8 (red).

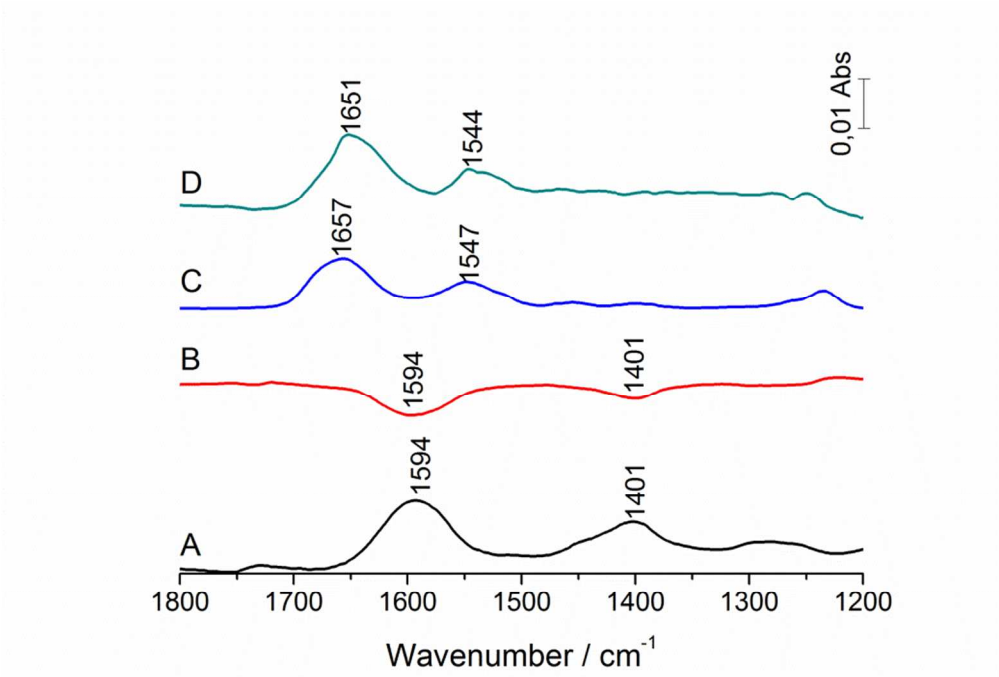

Figure S3. Characterization by SEIRAS of the different modification steps (A) Gold nanoparticles (B) mercaptohexanol + hexanethiol (C) Cytochrome aa_3 oxidase (D) Cytochrome ba_3 oxidase.

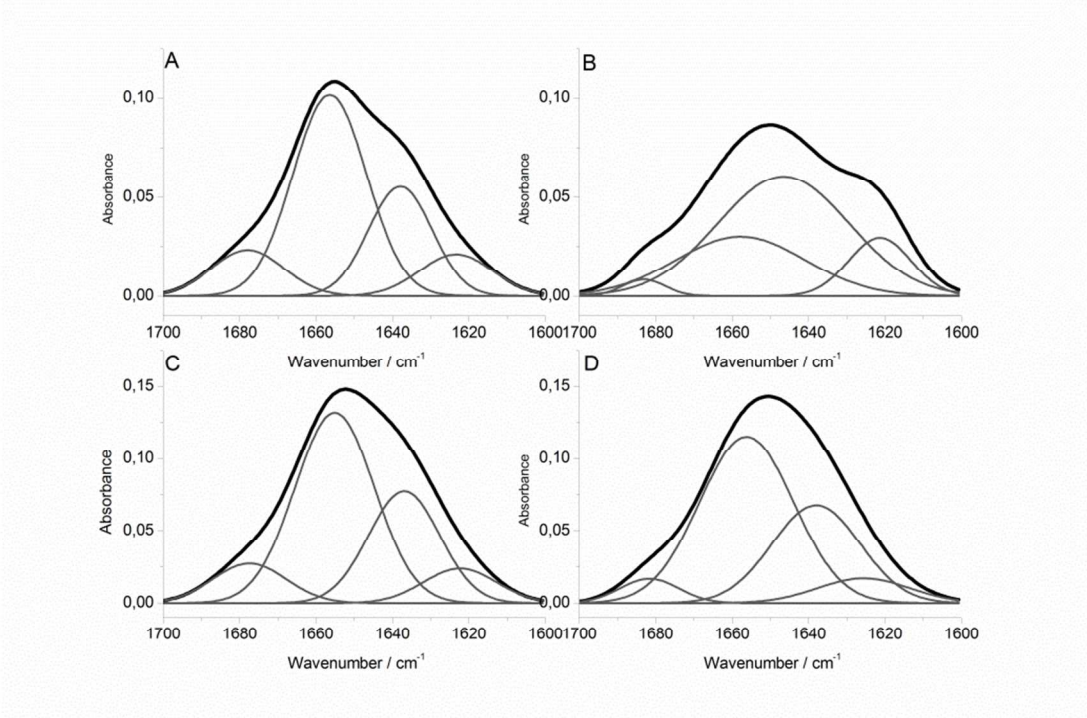

Figure S4. Deconvolution of the Amide I band of cytochrome aa_3 from *P. denitrificans* at 15°C (A) and 75°C (B) and cytochrome ba_3 from *T. thermophilus* at 15°C (C) and 75°C (D).

pH	Cytochrome aa_3 oxidase	Cytochrome ba_3 oxidase
6.5	-0.10	0.22
7	-0.12	0.20
7.5	-0.12	0.06
8	-0.12	0.02
8.5	-0.15	-0.02

Table S1. Catalytic potential obtained for both proteins at different pHs (in V).

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